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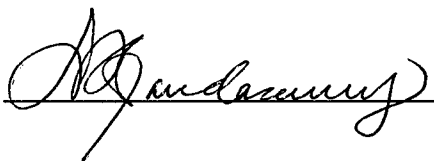
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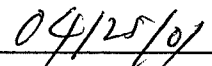
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13. ABSTRACT (<i>Maximum 200 Words</i>) The focus of this research is to generate and characterize novel human antibody fragments (Fabs) and peptides that bind breast cancer cells. A combinatorial approach is being taken to generate immunoglobulin Fab that recognize a specific determinant on breast cancer cells including the breast cancer-associated Thomsen-Friedenreich (T) glycoantigen. In addition, human Fab and peptides will be generated from combinatorial libraries that bind breast cancer cells, irrespective of defined cell surface markers. The hypothesis that is implicit in these studies is that combinatorial approaches will provide Fab and peptides specific to either defined or ill-defined antigens present on breast cancer cells. The goals of this study are to: 1) generate human IgG Fab that bind specifically to the T antigen expressed on breast cancers from pre-existing Fab, 2) isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial phage Fab display libraries, and 3) isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries. Results suggest phage libraries can yield Fab and scFv that bind to T antigen. Peptides and Fab that recognize lobular and ductal breast cancer cells are presently being sought.				
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**Combinatorial Approach to the Isolation of Human Antibody Fragments and Peptides to Breast
Carcinomas**

Year Two Annual Report

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(4) INTRODUCTION

Continued basic research in breast cancer is of fundamental importance since breast cancer is the leading cause of death in women (1). Breast cancer is known to develop from normal epithelium through several stages. It is not certain exactly when malignancy begins, however "invasive" breast carcinoma with metastatic potential occurs when epithelial cells invade the surrounding stroma (2). Invasive breast carcinoma may be preceded by noninvasive ductal or lobular hyperplasia or carcinoma. Preinvasive lesions as well as invasive breast tumors can often be detected by Mammography. Mammography has not been as reliable for the detection of these lesions in women with dense breast tissue, found commonly in pre-menopausal women. Therefore, a significant percentage of women may go undiagnosed by mammography. Alternative detection, diagnostic, and therapy regimens would be facilitated by reagents that can easily and sensitively detect breast cancer cell markers. The hypothesis that is implicit in these studies is that combinatorial human IgG and peptide libraries will provide molecules specific to antigens present on breast cancer cells. The Fab and peptides obtained will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer that may also facilitate early detection and diagnosis. The peptides and Fab that bind breast cancer cells, once radiolabeled, may be developed into important new therapeutic or imaging reagents. Novel approaches will be employed to generate new and improved human Fab that specifically recognize breast cancer cells. Fab immunoglobulin that recognize the well-documented breast cancer-associated Thomsen-Friedenreich (T) glycoantigen will be obtained. In addition, a combinatorial approach will be taken to isolate human Fab against breast cancer cells, regardless of detailed knowledge of target antigens. Combinatorial methods will also be applied for the isolation of small peptides that bind breast cancer cells. Both peptides and Fab that bind breast cancer cells are sought because it is difficult to predict which type of molecule will possess the most ideal properties for a diagnostic or therapeutic.

(5) BODY

The relevance of our proposed research is reflected in our goals of obtaining Fab and peptides that specifically and tightly bind breast cancer cells or cancer antigens. A limited number of murine monoclonal antibodies have been generated by others to the few known breast cancer-associated antigens, although none of them are cell and carcinoma specific. Moreover, the well documented immunogenicity and clearance problems of murine antibodies necessitates the development of 1) specific human Fab for application to human breast cancer and/or 2) the development of small molecule breast cancer-targeting agents. Our progress in the first year is summarized below.

Technical Objective 1: Generate human IgG Fab that bind specifically to the T antigen.

Task 1: Months 1-6: Generation of T Antigen Specific Fab by CDR Random Mutagenesis. Sequence Fab, and generate a database of T-binding sequences.

Research Accomplished: Discussed in year one progress report. Human IgG random CDR combinatorial Fab phage display libraries were screened for Fab that bound T antigen presented as a BSA-T antigen conjugant. After four rounds of affinity selection, individual phage clones were plated out and analyzed for antigen binding activity by filter binding assays. Soluble Fab were produced from clones exhibiting the strongest signal in the filter binding assays. The antigen binding affinities and specificities of the soluble Fab clones were determined by enzyme-linked immunosorbent assay (ELISA). Six Fab DNA clones (9, 17, 22, 52, 53, 54) with the highest ratio of asialofetuin to BSA binding properties were DNA sequenced. Analysis of the H chain sequence revealed that 5 out of the 6 clones had the same H chain DNA sequence. Clone 52 had a different heavy chain sequence but its CDR3 was homologous to the other clones. The L chains of the Fab were all different from each other, however.

Phage

9,17,22,53,54
52

Sequence

Val-Tyr-Cys-Ala-Lys-Met-Val
Tyr-Tyr-Cys-Ala-Lys-Met-Arg

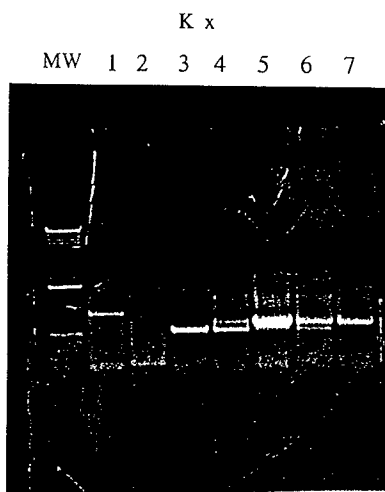
These results suggest a common antigen recognition motif. The isolation of 5 Fab with identical heavy chain sequences but different light chains indicates successful enrichment of Fab during the affinity selection process. Sequencing of more clones confirmed this pattern.

The naive human scFv phage display library was also screened for antibody fragments that bound the carcinoma-associated T antigen. The human scFv library has a diversity of 1×10^{10} and several antibody fragments with nanomolar affinities have been isolated from it (6). The phage clones were screened for antigen binding by ELISA. One scFv was expressed in *E. coli*, purified and re-examined for binding. Its specificity to T antigen appeared to be dependent on the protein rather than the carbohydrate component.

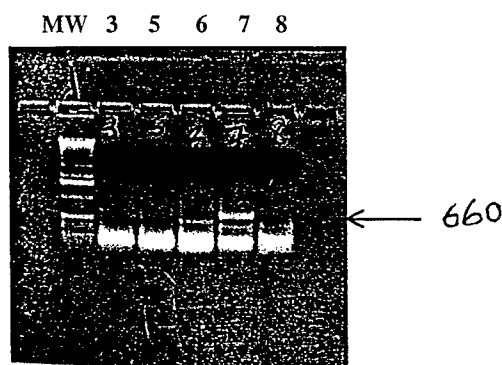
Task 2: Months 6-9: Isolate anti-T IgG from murine hybridomas and isolate human IgG from human sera or phage display libraries.

Research Accomplished: It was originally proposed to isolate IgG hybridomas obtained from collaborator George Springer. Unfortunately, he died recently and all cell lines and hybridomas were bequeathed to a senior investigator who will no longer make them available. As an alternative, we started the production of anti-T hybridomas in mice through injection of cancer cell lines expressing T antigen on their surfaces. Initial screening did not yield T-specific IgG. However, murine anti-T IgG3 was acquired from a new collaborator Kate Rittenhouse-Diakun. A hybridoma cell line producing the anti-T antibody (f11) was obtained and propagated. Cells were harvested and RNA was isolated. Murine IgG and kappa light chain primers were used on generated cDNA to clone the respective Fab genes (Figure 1). Two major PCR products were generated for the light chain and only one set of primers yielded the expected 600-700 base pair fragment of the heavy chain.

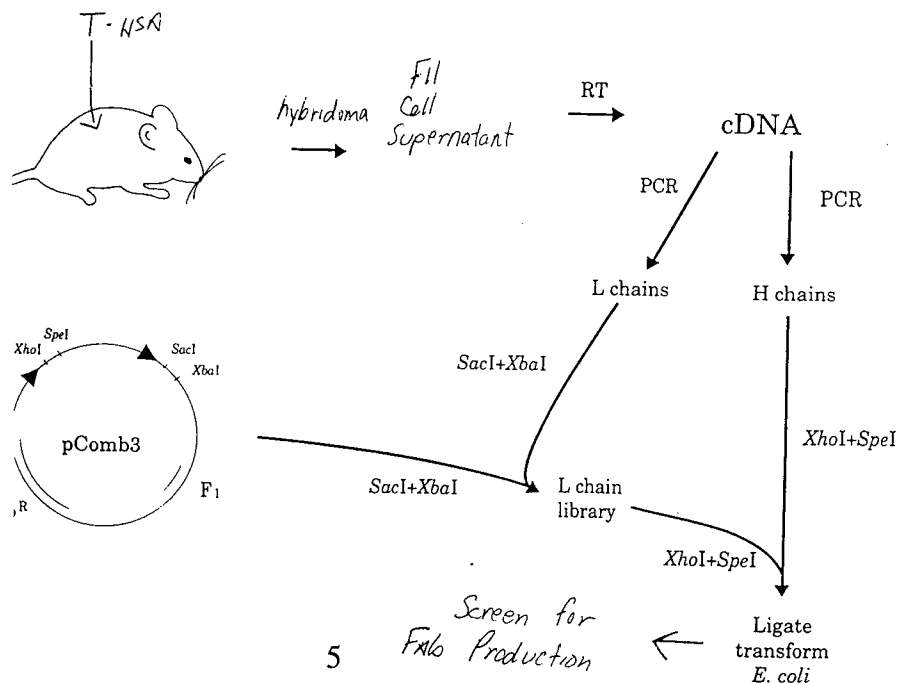
L Chain PCR of F11



H Chain PCR of F11



The PCR products were purified, restriction digested, and sub-cloned into the phagemid expression vector pComb 3 (see Figure 2).



The light and heavy chain DNA and corresponding amino acid sequences were determined. The complementarity-determining regions (CDRs) that dictate binding ability are shown in Figure 3.
Figure 3: Deduced Amino Acid Sequence of F11 Anti-T Antibody CDRs.

<u>VH</u>	<u>CDR1</u>	<u>CDR2</u>	<u>CDR3</u>
F11	TYWMH	FISPTDYTEYNKFRD	SFIGYNPDF
		(P30) FILP W WYAFSRI	
MK5	KYWMH	YINPSSG YTDYNQKFKG	SAYYRSFDY
DNA-1	SYVMH	YINPYND GTKYNEKFKG	GGYRPYYAMDY
<u>VL</u>			
F11	QASISCRSSQTI	KVSNRFS	FQGSHPFT
MK5	SASSSVSSRFLH	DTSKLAP	HQWSSYPLT
DNA-1	RASENIYSYLA	NAKTLAE	QHGYGTPLT

We have begun expression in *E. Coli*, but so far recombinant f11 appears to be poorly expressed.

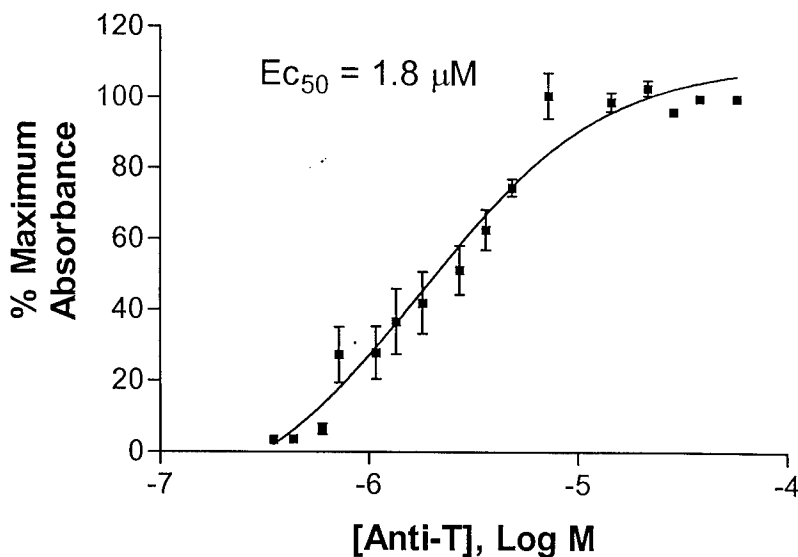
Task 3: Months 9-12: Evolution of a T Antigen-Specific Fab by DNA shuffling. Sequence Fab, express Fab in *E. coli*.

Research Accomplished: We have begun transplanting the murine variable region of anti-T f11 into a human Fab that expresses well. More emphasis will be placed on this task if expression of recombinant f11 in *E. coli* becomes problematic.

Task 4: Months 12-18: Purify Fab using affinity chromatography and examine binding to T antigen by ELISA, immunofluorescence and fluorescence titration.

Research Accomplished: We expressed four anti-T soluble scFv in *E. coli*. The scFv were secreted into both the media and the periplasm. Soluble scFv were produced from phage clones exhibiting the best ELISA results. The assays yield indicated the scFv were not specific for T antigen and could bind other antigens as well. We have spent months 12-18 purifying anti-T f11 antibody to homogeneity. The Fab recognizes the GalB1-3GalNac moiety of T and not other carbohydrates tested. ELISA results indicate the antibody has a high nanomolar affinity to T displayed on asialofetuin (ASF). See Figure 4.

Figure 4: ELISA of Anti-T Antibody Binding to ASF



We are in the process of performing fluorescence quenching with the anti-T Mab. Thus far, quenching data yields a similar dissociation constant to that derived from ELISA.

Technical Objective 2: Isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial Fab display libraries.

Task 1: Months 1-2: Obtain cell lines, grow cells, make frozen stocks and extracts.

Research Accomplished: Discussed in year one progress report. MDA-MB 435, MDA-MB-468 - lobular breast cancer cell lines, Hs578T a ductal cancer cell line, and Hs578Bstneg a normal ductal cell line were purchased.

Task 2: Months 3-12: Affinity select phage Fab libraries against cell lines.

Research Accomplished: We have performed affinity selections and will back select the final rounds with normal breast cancer cell lines.

Task 3: Months 13-16: Identify clones that bind cell lines using colony hybridization.

Research Accomplished: We have analyzed fifty clones to see if they bind specifically to breast cancer cells. So far phage that bind only to breast cancer cell lines and not normal cell lines have not been obtained. Therefore, we have taken our final round of phage and back selected them against normal cell lines. These phage clones are being analyzed.

Task 4: Months 17-24: Sequence Fab genes, express Fab and purify using affinity chromatography, and characterize binding using immunofluorescence and fluorescence titration.

Research Accomplished: We have sequenced twenty representative phage inserts from the different cell line panning experiments. A general consensus sequence has not been defined. Hence, more phage will be screened (task 3) and sequenced. It may be necessary to utilize more stringent conditions or perform more rounds of selection to increase our chances of finding consensus motifs. ELISA assays with the twenty clones did not yield specific binders for only cancer cells.

Technical Objective 3: Isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries.

Task 1: Months 25-28: Affinity select phage peptide libraries against cell lines.

Research Accomplished: Each of the cell lines was selected against a random 15mer phage peptide display library. Four rounds of affinity selection have been completed. We are ahead of schedule on this aim because it took longer to garner antibody for technical objective 1. This objective does not rely on the anti-T antibody.

Phage (approximately 10^{10} - 10^{12} virions) from a phage display library displaying random 15-mer peptides on its pIII coat protein, were added to each dish of cells containing growth media. Affinity selection was performed similar to that for the Fab libraries outlined in Task 2, Objective 2.

Following the wash stages, specifically bound phage were eluted with resuspension and gentle agitation. A small portion of the eluted phage was used to produce serial dilutions of the phage which were subsequently used to infect K91 Blue Kan *E. coli* cells. Cells were then plated on agar plates containing kanamycin and tetracycline - K91 Blue Kan cells carry resistance to kanamycin and the phage that infect the cells will confer tetracycline resistance to those cells. A portion of the first round eluted phage was used to infect *E. coli* cells which were subsequently used to produce phage particles to be used as the input phage for biopanning round #2. We have finished four rounds of selection with MDA-MB 435 and MDA-MB-468 breast cancer cell lines. We will back select the last eluates with normal cells to increase our chances of finding cancer-specific phage.

Task 2: Months 29-30: Characterize binding using immunofluorescence and fluorescence titration. To be done. We will also perform immunoblots, cell binding and ELISAs as initial screening tests.

Task 3: Months 31-36: Chemically synthesize selected peptides and determine binding properties to breast cancer cell lines. To be done.

(6) KEY RESEARCH ACCOMPLISHMENTS

- * Affinity Selection of Random HCDR Fab and scFv libraries Against T Antigen.
- * Determine DNA and Amino Acid Sequence of 5 Fab clones.
- * Purify anti-T scFv and Examine Binding by ELISA.
- * Purify anti-T monoclonal antibody f11.
- * Determine affinity of anti-T antibody to T-containing asialofetuin using ELISA and fluorescence quenching.
- * Isolate mRNA from f11 hybridoma and generate cDNA.
- * Clone via PCR the heavy and light chain Fab genes of f11.
- * Determine the DNA and encoded amino acid sequence of the f11 Fab.
- * Subclone the heavy and light chains of f11 into E. Coli expression vector pComb3, begin expression studies.
- * Screen Antibody and Peptide libraries for Molecules that Bind Lobular and Ductal Breast Cancer Cell Lines.
- * Study role of T antigen in cell adhesion.

(7) REPORTABLE OUTCOMES

- * Clone and sequence anti-T IgG3 heavy and light chains.
- * DOD Breast Cancer Postdoctoral Fellowship Awarded to Mark Meighan, a postdoctoral fellow in my laboratory.
- * Attend ERA of Hope meeting in Atlanta and present poster on our work.
- * Co-author manuscript on the role of T antigen in adhesion. "Glinsky, V.V., Huflejt, M.E., Glinsky, G.V., Deutscher, S.L., and Quinn, T.P. (2000) Effects of T Antigen Specific Peptide P-30 on β -Galactoside Mediated Homotypic Aggregation and Adhesion to the Endothelium of MDA-MB-435 Human Breast Carcinoma Cells. Cancer Res. 60, 2584-2588."

(8) CONCLUSIONS

Alternative detection, diagnostic, and therapeutic approaches are needed to help reduce the morbidity and mortality of breast cancer. Our approach is to employ combinatorial human Ig and peptide libraries to generate molecules specific to antigens present on breast cancer cells. The Fab and peptides will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer, that once radiolabeled, may facilitate early detection and diagnosis. Human IgG random CDR combinatorial Fab phage display libraries and more diverse scFV libraries were screened for Fab that bound the breast cancer-associated T antigen presented as a BSA-T antigen conjugant or asialofetuin. Numerous Fab and scFv-displaying phage were selected in this procedure and analyzed for binding to T antigen using ELISA. Most of the scFv did not bind specifically to the T epitope. We have obtained a new anti-T monoclonal antibody that we have determined binds specifically to the T carbohydrate. The H and L chain genes have been cloned using a diverse set of immunoglobulin primers. The DNA and encoded sequence of the recombinant Fab suggests the anti-T antibody to be rather unique. Its heavy chain is of a rare subclass. The recombinant Fab will be expressed in E. coli and analyzed further (5). We are shuffling CDRs of a non-specific Fab in the hopes of converting it to a T-binding antibody. These problems can also be addressed through the simultaneous screening of peptide libraries for molecules that bind T antigen or breast cancer cells since peptides may be as or more valuable as diagnostic or therapeutic agents. We suggest that affinity selection of

peptide libraries can begin earlier than originally planned since breast cancer cell lines that are currently in use in Fab library screening can also be employed in this approach.

"So What Section"

-Recombinant antibody fragments that bind T antigen may be useful reagents in breast cancer diagnosis, detection, or localization.

-Recombinant antibody fragments can be engineered to contain a radiometal chelation sequence such that radiolabeling with Re186/188 can yield radiopharmaceuticals that could destroy cancer cells.

-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to identify new breast cancer antigens.

-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens.

-Peptides that bind breast or ductal breast cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens. Peptides may be tolerated in higher doses in patients than antibodies.

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